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NKX3.1 Is Regulated by Protein Kinase CK2 in Prostate Tumor Cells

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Diminished expression of NKX3.1 is associated with prostate cancer progression in humans, and in mice, loss of *nkx3.1* leads to epithelial cell proliferation and altered gene expression patterns. The NKX3.1 amino acid sequence includes multiple potential phosphoacceptor sites for protein kinase CK2. To investigate posttranslational regulation of NKX3.1, phosphorylation of NKX3.1 by CK2 was studied. In vitro kinase assays followed by mass spectrometric analyses demonstrated that CK2 phosphorylated recombinant NKX3.1 on Thr89 and Thr93. Blocking CK2 activity in LNCaP cells with apigenin or 5,6-dichlorobenzimidazole riboside led to a rapid decrease in NKX3.1 accumulation that was rescued by proteasome inhibition. Replacing Thr89 and Thr93 with alanines decreased NKX3.1 stability in vivo. Small interfering RNA knockdown of CK2α′ but not CK2α also led to a decrease in NKX3.1 steady-state level. In-gel kinase assays and Western blot analyses using fractionated extracts of LNCaP cells demonstrated that free CK2α′ could phosphorylate recombinant human and mouse NKX3.1, whereas CK2α′ liberated from the holoenzyme could not. These data establish CK2 as a regulator of NKX3.1 in prostate tumor cells and provide evidence for functionally distinct pools of CK2α′ in LNCaP cells.

As the second leading cause of cancer deaths and the most frequently diagnosed malignancy in men, prostate cancer presents significant challenges from the perspective of both clinical and molecular oncology (28). As with all malignancies, the characterization of genetic changes associated with disease progression is a high priority. Human chromosome 8p has long been suspected to harbor one or more tumor suppressor genes involved in the etiology of prostate cancer based on analyses of allelic loss (7). Loss of heterozygosity of 8p21 is observed in a high percentage of intraepithelial prostatic neoplasias and early carcinoma lesions, strongly implicating this region in the initial stages of prostate carcinogenesis (18, 19, 25, 54).

A substantial body of evidence points to an NK class homeobox gene as a leading 8p21 candidate for a prostate growth-regulatory gene involved in tumor initiation (48). Identified in mice as a prostate-restricted and androgen-regulated gene (9, 26, 43, 47, 48), *nkx3.1* and its human homolog, *NKX3.1*, have been extensively characterized at the genetic level for links to prostate cancer. In humans, *NKX3.1* maps to the minimal region of overlap among 8p21 loss-of-heterozygosity cases that have been analyzed to date (51).

The Knudson two-hit model predicts that if *NKX3.1* behaves as a classical tumor suppressor gene, then in prostate cancer cases with 8p21 loss, the remaining allele should incur an inactivating mutation (32). Several studies have demonstrated that in such cases the remaining *NKX3.1* allele is wild type and is transcribed, arguing against a canonical tumor suppressor function (41, 55). However, a tumor suppression role for *NKX3.1* is easily reconciled with more recent interpretations of tumor suppressor gene function that highlight the need to consider haploinsufficiency (16, 44, 45). It is becoming increasingly clear that gene dosage effects among many tumor suppressors, including *p53* (53) and perhaps even *Rb* (61), can play an important role in the development of malignancy. A current model proposes that a loss-of-function mutation in a tumor suppressor gene confers a growth advantage that results in an expansion of cells carrying that initiating mutation (44). This scenario increases a target cell population in which the next of an estimated total of four to eight genetic changes required to support the evolution of a malignant state can occur.

The importance of NKX3.1 as a dose-dependent regulator of prostate epithelial cell growth is strongly supported by analyses of *nkx3.1* knockout mice (3, 8, 46, 52). Homozygous *nkx3.1* mutant mice develop prostate epithelial hyperplasia and dysplasia that progresses with age (8, 46, 52), and lesions with histologic features strongly resembling human prostatic intraepithelial neoplasia develop in homozygous mice between 1 and 2 years of age (30). Importantly, both hyperplasia and prostatic intraepithelial neoplasia-like lesions also occur in a significant proportion of *nkx3.1* heterozygous mutants (30). In conjunction with loss of one allele of the tumor suppressor gene *pten*, loss of one allele of *nkx3.1* leads to high-grade prostatic intraepithelial neoplasia capable of progressing to invasive adenocarcinoma that metastasizes to lymph nodes (1, 31). Concomitant loss of a $p27^{kip1}$ allele exacerbates the phenotype observed in *pten*/*nkx3.1* double heterozygotes (22).

Using a castration-regeneration system with *nkx3.1* knockout mice, a battery of genes that are coregulated by androgens in an *nkx3.1* dosage-dependent manner have been defined (38). These genes exhibit a range of sensitivities to *nkx3.1* dosage, with some being shut off upon loss of one allele, while others are less dramatically altered. This study also clearly implicates *nkx3.1* in growth control by demonstrating that in heterozygous *nkx3.1* knockout mice, the exit of prostate epithelial cells from the cell cycle is delayed, resulting in a sharp increase in cell number.

Immunohistochemical analyses of prostatic intraepithelial neoplasia lesions and prostate tumors have demonstrated that

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diminution or complete loss of NKX3.1 expression is a common event (11, 31). Curiously, several studies have shown that *NKX3.1* mRNA levels do not diminish in tumors and may in fact increase with disease progression (41, 57) although concordance between *NKX3.1* mRNA and protein has been reported in one recent study (33). These paradoxical and conflicting observations underscore the need to investigate the regulation of NKX3.1 at multiple levels.

NKX3.1 is known to be a phosphoprotein, and phosphorylation has been shown to alter its DNA binding affinity in vitro (23). We initiated an investigation of NKX3.1 regulation focusing on posttranslational regulation by phosphorylation. In the work reported here, we have demonstrated that CK2 can phosphorylate NKX3.1 in vitro, shown that Thr89 and Thr93 are CK2 phosphoacceptor sites, and determined that protein kinase CK2 regulates the half-life of NKX3.1 in prostate tumor cells. We have also determined that NKX3.1 is degraded primarily through a proteasomal pathway, suggesting that phosphorylation by CK2 protects NKX3.1 from degradation. Our studies further indicate that a specific isoform of the CK2α′ catalytic subunit of CK2 is responsible for phosphorylating NKX3.1, providing new insights into the regulation of CK2 activity. Together, these observations establish a strong link between NKX3.1 and CK2 and may implicate CK2 in prostate malignancy.

MATERIALS AND METHODS

NKX3.1 purification. Recombinant human NKX3.1 was expressed in bacteria and purified as described (14). Mouse Nkx3.1 cloned in pPROEX-hta (Invitrogen, Carlsbad, CA) was transformed into *Escherichia coli* BL21 cells and induced at 37°C with 0.8 mM isopropylthiogalactopyranoside (IPTG). Cells were collected and resuspended in water and broken at 1,000 lb/in^2 in a French press. After centrifugation at 20,000 × *g* for 30 min the pellet was dissolved in 5 ml urea lysis buffer (8 M urea, 100 mM NaH_2PO_4, 20 mM imidazole, pH 7.5) per 500 ml of culture volume. The lysate was centrifuged at 20,000 × *g* for 30 min, and Nkx3.1 present in the supernatant was loaded to a nickel column and eluted with 8 M urea, 100 mM NaH_2PO_4, 250 mM imidazole, pH 7.5.

In vitro kinase assay and isoelectric focusing of NKX3.1. To phosphorylate recombinant human NKX3.1 with CK2, 500 units of CK2 holoenzyme (New England Biolabs, Beverly, MA) was incubated at 25°C for 1 h with 300 pmol of recombinant human NKX3.1 in a 30-μl reaction volume containing 1× CK2 reaction buffer (New England Biolabs, Beverly, MA) supplemented with 200 μM ATP. The reaction was stopped by precipitating proteins with trichloroacetic acid. The pellet was resuspended in isoelectric focusing buffer containing 7 M urea, 2 M thiourea, 1% C7BzO, and 0.1% immobilized pH gradient (IPG) buffer, pH 3 to 11 NL (Amersham Biosciences, Piscataway, NJ) and applied to IPG strips (7-cm, pH 3 to 10; Bio-Rad, Hercules, CA). The IPG strip was focused on an isoelectric focusing cell (Bio-Rad, Hercules, CA) using a program as follows: 250 V, linear gradient, 20 min; 4,000 V, linear gradient, 2 h; and 4,000 V, rapid gradient, 11,000 Vh. After second-dimension electrophoresis in 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gels, proteins were stained using Coomassie brilliant blue R-250.

Mass spectrometry. Gel spots were excised and destained with 50 mM NH_4HCO_3/50% acetonitrile. Gel plugs were then dehydrated with 50 μl acetonitrile and dried completely in a speed vacuum. Gel plugs were then rehydrated in 25 mM NH_4HCO_3 containing 200 ng trypsin (Promega, Madison, WI). Digestion was performed at 37°C overnight in a shaker. The tryptic peptides were desalted and concentrated using a μC18 ZipTip (Millipore, Bedford, MA). The peptides were directly spotted onto a target plate from the ZipTip by eluting with 0.75 μl elution buffer containing 5 mg/ml α-cyano-4-hydroxycinnamic acid (CHCA) (Sigma, St. Louis, MO), 5 mM $NH_4H_2PO_4$ in 60% acetonitrile/0.1% trifluoroacetic acid. $NH_4H_2PO_4$ was included to suppress matrix signals, reduce noise, and enhance detection of phosphopeptides (49, 59, 62). Positive-ion matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra were acquired using an Autoflex mass spectrometer (Bruker Daltonics, Billerica, MA) operated in linear mode. The potential phosphorylated peptides were selected as precursor ions for post-source-decay (PSD) analysis. The MALDI-PSD spectra were obtained with a parent ion selection of ±30 Da. The spectra were acquired in 16 segments under computer control and stitched together using the XMASS software (Bruker Daltonics, Billerica, MA).

Cell culture. LNCaP cells were cultured in RPMI 1640-10% fetal bovine serum with 5% CO_2 in air at 37°C in a humidified chamber. Where indicated, 60 μM 5,6-dichlorobenzimidazole riboside (DRB) (Calbiochem, Cambridge, MA), 75 μM apigenin, 10 μM MG132, 1 μM epoximicin, or 10 μM cycloheximide was administered by diluting from stocks prepared in dimethyl sulfoxide.

LNCaP cell fractionation. LNCaP cells at 80% confluence were harvested and lysed in 150 mM NaCl, 50 mM Tris (pH 7.5), 0.25% NP-40 containing a protease inhibitor cocktail (Roche, Indianapolis, IN). After centrifugation at 10,000 × *g* for 15 min the supernatant was passed through a 0.45-μm filter; 50 mg lysate was dialyzed against 50 mM diethanolamine, pH 8.8, and loaded onto a 1-ml Mono Q column (Amersham Biosciences, Piscataway, NJ) at 4°C. The column was washed with 3 ml of 50 mM diethanolamine, pH 8.8, and eluted with a 20-ml NaCl gradient from 0 to 0.5 M followed by 5 ml 1 M NaCl, and 1-ml fractions were collected and analyzed by an in-gel kinase assay and Western blotting.

In-gel kinase assay. In-gel kinase assays were performed essentially as described (56) in the presence of 1 μCi/ml [γ-^{32}P]ATP (3,000 Ci/mmol) without the addition of unlabeled ATP; 0.5 mg/ml recombinant NKX3.1 or Nkx3.1 was polymerized in 12% polyacrylamide gels.

Expression constructs, siRNA, and LNCaP cell transfection. The hemagglutinin (HA)-tagged NKX3.1 (pcDNA3-NKX3.1-HA) and His-tagged ubiquitin expression vectors have been described (15, 40). The NKX3.1 T89A/T93A and S150A mutants were generated using a QuikChange site-directed mutagenesis kit (Stratagene, San Diego, CA) according to the manufacturer's instructions. The oligonucleotides used to generate the T89A/T93A mutant were 5′-GAGGAGGCC GAGGCCCTGGCAGAGGCCGAGCCAGAAAGG-3′ and 5′-CCTTTCTGGC TCGGCCTCTGCCAGGGCCTCGGCCTCGGCCTCCTC-3′; the oligonucleotides used to generate the S150A mutant were 5′-CATCAGAAGTACCTGGCCG CCCCTGAACGGGCC-3′ and 5′-GGCCCGTTCAGGGGCGGCCAGGTACTT CTGATG-3′. Plasmids were transiently transfected into LNCaP cells using Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions; 24 h after transfection, pharmacologic agents were applied as described in the text.

Small interfering RNA (siRNA) oligonucleotides were purchased from Dharmacon (Chicago, IL). The sequence for CK2α′ was 5′-CAGUCUGAGGAGCC GCGAGdTdT (58), and that for CK2α was 5′-AACAUUGAAUUAGAUCCA CGUdTdT. The negative control siRNA was the siCONTROL nontargeting siRNA pool (catalog no. D-001206-13-05, Dharmacon, Chicago, IL). The siRNA oligonucleotides were transfected into LNCaP cells using a double transfection method (6, 37). Briefly, 5 μl of 20 μM siRNA oligonucleotides and Lipofectamine 2000 were diluted into 100 μl serum-free RPMI medium for 5 min and then mixed for an additional 20 min. During the 20-min incubation, cells grown to 80% confluence were trypsinized, spun down at 100 × *g*, washed once in phosphate-buffered saline, and resuspended in serum-free RPMI medium. The Lipofectamine-siRNA complex (200 μl) was then mixed with 6×10^5 cells/transfection and plated onto one well of a six-well plate, in a final volume of 1 ml (final siRNA concentration: 100 nM). After 5 h, the transfection mixture was removed and complete serum-containing medium was added; 24 h later, the attached cells were transfected again using 100 nM siRNA and a standard transfection procedure. The cells were harvested for analysis at 48 h after initial transfection.

In vivo ubiquitination assay. LNCaP cells in a six-well plate were cotransfected with NKX3.1, the His-ubiquitin expression vector, or empty pcDNA3.1 vector as described above. Twenty-four hours after transfection, the cells were treated with 10 μM MG132 for an additional 12 h. After washes in phosphate-buffered saline, the cells were directly lysed in the urea lysis buffer and cleared by centrifuge. The lysates were then incubated with 30 μl nickel resin for 30 min at room temperature. After extensive washes using the urea lysis buffer, bound proteins were analyzed by boiling the resin in SDS-PAGE loading buffer, followed by SDS-PAGE and immunoblotting using anti-NKX3.1 antibodies.

Western blot analysis. LNCaP cells were lysed in 150 mM NaCl, 50 mM Tris, 0.25% NP-40, pH 7.5, and protein concentration was determined using Bio-Rad protein assay reagents (Bio-Rad Laboratories, Hercules, CA). Identical amounts of total protein were loaded in each lane of a 12% SDS-PAGE gel and transferred to polyvinylidene difluoride membranes after electrophoresis. Western blot analyses were performed using the following antibodies and dilutions: rabbit anti-NKX3.1 (1:5,000) (C. J. Bieberich and X. Li, unpublished data), mouse anti-β-tubulin clone E7 (1:1,000) (M. Klymkowski, Developmental Studies Hybridoma Bank, University of Iowa, Department of Biological Sciences, Iowa City, Iowa); mouse anti-β-actin clone AC74 (1:10,000) (Sigma, St. Louis, MO); rabbit

anti-CK2α′ (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-CK2α (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-CK2β (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA); and rat anti-HA (1:500) (Roche, Indianapolis, IN). Signal intensities on films were quantified using ImageQuant 5.2 software (Molecular Dynamics, Sunnyvale, CA).

RESULTS

CK2 phosphorylates NKX3.1 in vitro. Interrogation of the NKX3.1 amino acid sequence with the Phosphobase 2.0 algorithm (34) revealed the presence of potential phosphoacceptor sites for multiple kinases including protein kinase CK2 (data not shown). CK2 is a ubiquitous kinase that has been implicated in the control of critical cellular functions, including proliferation and apoptosis (4, 36, 42). CK2 kinase activity is mediated by two distinct catalytic subunits termed CK2α and CK2α′. NKX3.1 contains three residues that fit the minimum CK2 consensus sequence (S/TXXD/E): Thr89, Thr93, and Ser150. To determine whether NKX3.1 could serve as a substrate for protein kinase CK2, an in vitro kinase reaction was performed using recombinant NKX3.1 and CK2 holoenzyme consisting of CK2α complexed with CK2β in the presence of γ-^{32}P-labeled ATP. Resolution of the products on an SDS-PAGE gel followed by autoradiography showed that NKX3.1 could be efficiently phosphorylated (data not shown).

To identify the site(s) of CK2 phosphorylation in NKX3.1, the products of a CK2 kinase reaction were resolved on a two-dimensional SDS-PAGE gel and stained. Unreacted NKX3.1 was run on a parallel two-dimensional gel. Coomassie blue staining revealed that the isoelectric point of essentially all of the NKX3.1 protein displayed an acidic shift in pH after the CK2 kinase reaction (Fig. 1A). The region of the gels containing CK2-reacted NKX3.1 and unreacted NKX3.1 were excised, in-gel digested by trypsin, and analyzed using a MALDI-TOF mass spectrometer in positive-ion linear mode. Comparison of the spectra from the putative phosphorylated and unreacted NKX3.1 spots revealed three peptides shifted by 80 or 160 Da that were specific for the CK2-reacted NKX3.1 (Fig. 1B).

The 1,902.80-Da peptide represents the diphosphorylated form of peptide ^{82}AAPEEAE**T**LAE**T**EPER^{97} (theoretical mass, 1,742.81), which contains two potential phosphoacceptor residues, Thr89 and Thr93 (bold), both of which are in the context of a canonical CK2 site. Ions that could correspond to the nonphosphorylated or monophosphorylated forms of amino acids 82 to 97 (theoretical mass, 1,742.81 and 1,822.79, respectively) were not observed in the CK2-reacted NKX3.1 spectrum, indicating that all of this peptide was phosphorylated on both threonine residues (Fig. 1B). MALDI-TOF mass spectrometry with post-source-decay analysis (Fig. 1C) of the 1,902.80-Da ion indicated that this peptide is the diphosphorylated form of the peptide carrying residues 82 to 97, since one and two neutral losses of HPO_3 from the parent ion were observed (59).

The other two specific ions, with *m*/*z* values of 1,394.58 and 2,009.99 Da, most likely represent the phosphorylated form of peptides ^{69}AGAQNDQLSTGPR^{81} and ^{98}HLGSYLLDSENTSGALPR^{115} (theoretical masses, 1,314.64 and 1,929.97, respectively). The monophosphorylation states of these two peptides (1,394.58 and 2,009.99 Da) were confirmed by PSD analyses (data not shown). These two peptides contain multiple serine or threonine residues that could serve as phosphoacceptors, although none are in the context of a typical CK2 site. Further analyses of the phosphorylation of these two peptides will be published elsewhere. An ion representing the phosphorylated form of the peptide ^{148}YLSAPER^{154} (theoretical mass, 835.43) containing Ser150 was anticipated but surprisingly was not observed. These data demonstrated that CK2 can phosphorylate NKX3.1 in vitro at two canonical CK2 sites (Thr89 and Thr93) in addition to at least two other sites.

Pharmacologic inhibition of CK2 activity alters the steady-state level of NKX3.1 in LNCaP cells. To determine whether a functional relationship exists between CK2 activity and NKX3.1 in prostate tumor cells, LNCaP cells were cultured in the presence of apigenin, a potent inhibitor of CK2 activity. Extracts of cells harvested after 2 or 4 h of apigenin exposure were analyzed by Western blot analysis to determine the level of NKX3.1. Within 2 h of apigenin exposure, the steady-state level of NKX3.1 was diminished by 60% and reached 90% after 4 h of apigenin exposure (Fig. 2A and B). Nearly identical results were obtained using the more selective small-molecule kinase inhibitor DRB (data not shown). Microscopic analysis of the LNCaP cells during the course of apigenin or DRB treatment did not reveal any morphological changes. These data suggest that CK2 activity plays a role in the maintenance of NKX3.1 expression at the protein level, mRNA level, or both in LNCaP cells.

To determine whether apigenin treatment altered the level of *NKX3.1* mRNA, Northern blot analyses were performed on RNA extracted from the same pools of apigenin-treated cells that were analyzed by Western blotting for NKX3.1. After 2 h of apigenin exposure, the level of *NKX3.1* mRNA was reduced by nearly 30%, and that diminished level was maintained at 4 h of exposure (Fig. 2C). Together with the Western blot analyses, these data demonstrate that inhibition of CK2 activity results in a significant decrease in the level of NKX3.1 in LNCaP cells and suggest that this effect is mediated at least in part by diminishing *NKX3.1* mRNA accumulation. However, the sharp decrease in NKX3.1 observed upon CK2 inhibition prompted us to determine if CK2 could be acting primarily at the protein level.

Mutation of CK2 phosphorylation sites alters accumulation of NKX3.1 in LNCaP cells. The effect of CK2 inhibition of NKX3.1 on the steady-state level of NKX3.1 could result from effects at the mRNA or protein level or both. If CK2 phosphorylation of NKX3.1 plays a direct role in maintaining stability, then mutation of potential CK2 phosphorylation sites would be predicted to result in a less stable NKX3.1 isoform.

Based on the in silico identification of potential CK2 phosphorylation sites and in vitro kinase assay data, a Thr89/Thr93 alanine substitution mutant (T89A/T93A) was generated to determine the effect of eliminating these potential phosphoacceptor sites on NKX3.1 stability. In a second mutant, the Ser150 residue which lies in the context of a CK2 consensus site but was not phosphorylated in vitro was also mutated to alanine (S150A). These phosphorylation site mutants were transiently transfected in triplicate into LNCaP cells, and HA-tagged wild-type NKX3.1 served as a control. Western blot analyses using antibodies against the HA tag to compare the steady-state level of the S150A and wild-type proteins expressed by the transfected genes revealed no difference in

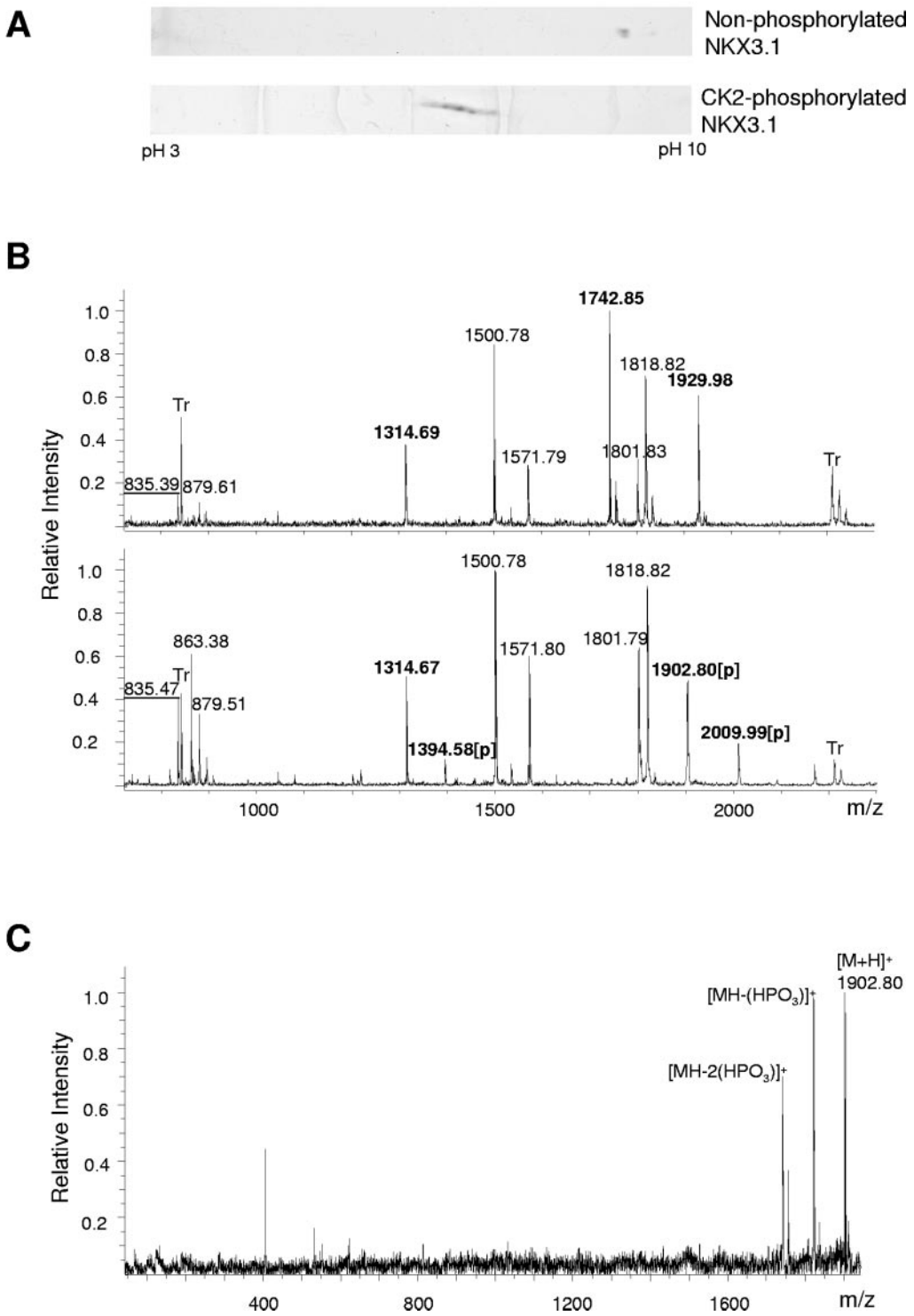

FIG. 1. CK2 phosphorylates NKX3.1 in vitro. (A) Two-dimensional PAGE analysis of recombinant NKX3.1 and CK2-phosphorylated NKX3.1. The gels were stained with Coomassie brilliant blue R-250. The NKX3.1 protein was shifted from pH ~9 to ~7 after the CK2 phosphorylation reaction. (B) MALDI spectra of the NKX3.1 protein in panel A acquired in linear positive mode using CHCA with ammonium phosphate as the matrix. Top panel: the spectrum for nonphosphorylated NKX3.1; bottom panel: the spectrum for CK2-phosphorylated NKX3.1. The *m/z* values of peaks corresponding to the tryptic peptides containing amino acids 69 to 81, 82 to 79, and 98 to 115 (theoretical masses: 1,314.64, 1,742.81, and 1,929.97 Da, respectively), as well as their phosphorylated forms (theoretical masses: 1,396.62, 1,902.77, and 2,009.95 Da, respectively) are in bold. The *m/z* values of the peptide corresponding to amino acids 148 to 154 (theoretical mass: 835.43 Da) that contains a consensus CK2 site is underlined. Tr denotes autotryptic fragments of the protease trypsin. Note the absence of peaks at 1,742.81 and 1,929.97 Da in the bottom spectrum. (C) MALDI-TOF PSD spectrum of the peptide of *m/z* 1,902.80 detected in panel B. The sequential losses of 80 Da from the parent ion indicate that the peptide of *m/z* 1,902.80 is diphosphorylated.

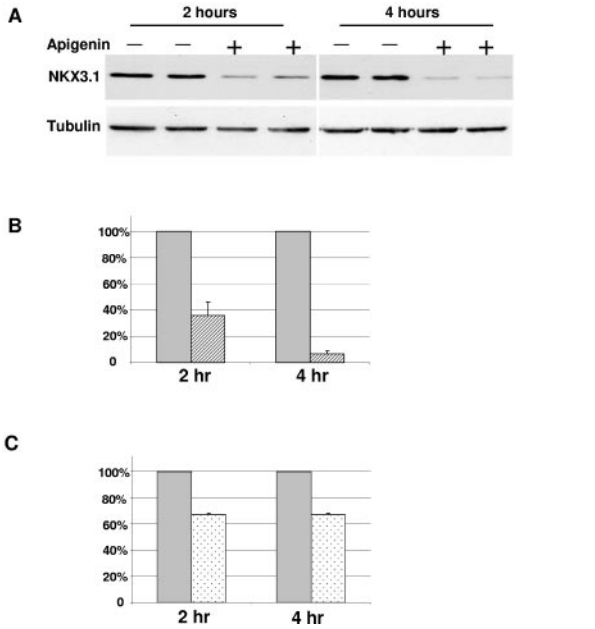

FIG. 2. CK2 blockade decreases the level of NKX3.1 in LNCaP cells. (A) Whole-cell lysates of LNCaP cells treated with apigenin were analyzed by Western blot analysis to detect NKX3.1 and β-tubulin. (B) Quantitative analysis of Western blots shown in panel A. NKX3.1 and tubulin bands were analyzed using the ImageQuant software. The relative NKX3.1 protein levels normalized to tubulin levels are graphed. Gray bar, dimethyl sulfoxide control; hashed bar, apigenin-treated samples. (C) Quantitative analysis of Northern blots to detect NKX3.1 mRNA in the same apigenin-treated LNCaP cells shown in panel A. Gray bar, dimethyl sulfoxide control; stippled bar, apigenin-treated samples.

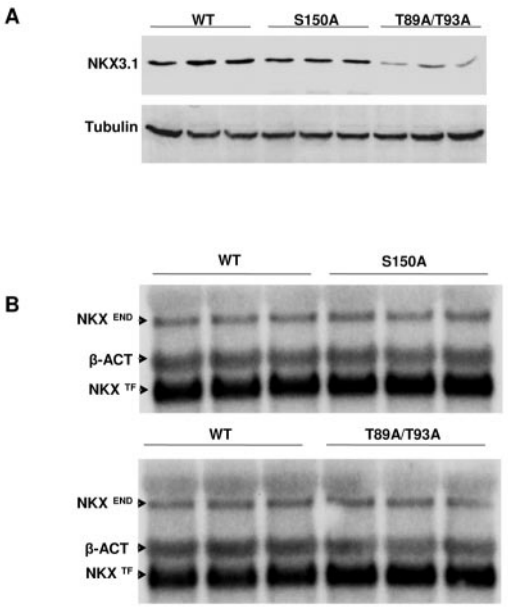

FIG. 3. Replacement of CK2 phosphorylation sites Thr89 and Thr93 with alanines reduces the steady-state level of NKX3.1 in LNCaP cells. (A) LNCaP cells were transfected in triplicate with wild-type (WT) NKX3.1 and the T150A and T89A/T93A alanine substitution mutants and harvested after 24 h for Western blot analysis of NKX3.1 using an anti-HA antibody to detect the transfected forms of NKX3.1. (B) Northern blot analysis to detect *NKX3.1* mRNA in the same transfected cells shown in panel A. NKX3.1^{END} denotes the position of endogenous NKX3.1 mRNA; NKX3.1^{TF} denotes the position of transfected *NKX3.1* mRNA.

accumulation (Fig. 3A). In contrast, the T89A/T93A mutant consistently showed a lower steady-state level than wild-type NKX3.1 (Fig. 3A). To ensure that the difference in accumulation was not due to a difference in mRNA levels, Northern blot analyses were performed using RNA from the same populations of cells analyzed by Western blot. No differences in mRNA levels among the transfected genes were observed (Fig. 3B). These data suggest that CK2 phosphorylation at Thr89 or Thr93 or both stabilizes NKX3.1.

To rule out the possibility that the diminished level of NKX3.1 (T89A/T93A) was due to a decrease in mRNA translation efficiency, the half-life of mutant and wild-type NKX3.1 protein was compared in transiently transfected LNCaP cells. Western blot analysis of NKX3.1 protein levels at 30-min intervals after blocking translation with cycloheximide revealed a half-life of approximately 80 min for wild-type NKX3.1, whereas the half-life of the T89A/T93A mutant was only 43 min (Fig. 4).

Blocking the proteasome reverses the effect of CK2 inhibition on NKX3.1 accumulation. The reduced half-life of the T89/T93 mutant suggested that CK2 may act to stabilize NKX3.1. To investigate this effect further, it was first necessary to determine the proteolytic pathway by which NKX3.1 is degraded in LNCaP cells. Treatment with MG132 or the potent and irreversible proteasome inhibitor epoximicin resulted in an increase in the protein level of NKX3.1 (Fig. 5A), suggesting that this protein is degraded by the 26S proteasome. In contrast, inhibition of either calpain or cathepsins did not alter the steady-state level of NKX3.1 (data not shown).

Typically, proteins degraded by the proteasome are first marked for destruction by polyubiquitination. To determine if NKX3.1 degradation is mediated by the ubiquitin-proteasome system, LNCaP cells were cotransfected with NKX3.1 and a His-tagged ubiquitin expression vector and treated with MG132 24 hours after transfection. After proteasome blockade, ubiquitinated proteins were enriched by nickel affinity chromatography and the resulting eluate was analyzed by Western blot using anti-NKX3.1 antibodies. Higher-molecular-weight isoforms of NKX3.1 were specifically enriched in the presence of His-ubiquitin (Fig. 5B), providing clear evidence that NKX3.1 is polyubiquitinated in LNCaP cells. Taken together, these data strongly suggest that NKX3.1 is degraded by the 26S proteasome.

To determine whether the effect of CK2 inhibition on the level of NKX3.1 in LNCaP cells could be mediated in part through a posttranslational mechanism, cells were treated with either apigenin or DRB and proteasomal activity was blocked with MG132. Western blot analyses revealed that treatment with MG132 resulted in a reversal of the diminution of NKX3.1 induced by CK2 inhibition (Fig. 5C). In cells that were cotreated with either apigenin or DRB and MG132, the level of NKX3.1 was essentially unchanged with respect to that in untreated cells. LNCaP cells were then transiently transfected with the HA-tagged wild-type and T89A/T93A phosphorylation mutant NKX3.1 expression constructs and treated with

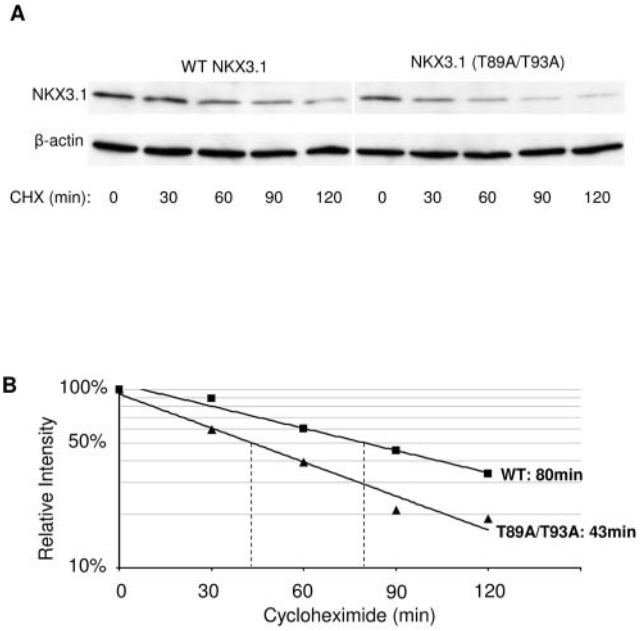

FIG. 4. Reduced half-life of the T89A/T93A mutant. (A) Western blot analysis of whole-cell extracts of LNCaP cells transfected with wild-type (WT) NKX3.1-HA or the T89A/T93A mutant. The cells were sampled at the indicated times after cycloheximide (CHX) treatment. The transfected forms of NKX3.1 were detected by an anti-HA antibody. (B) Quantification of Western blot data in panel A. The NKX3.1 levels were normalized to that of β-actin and graphed on a semilog plot. The calculated half-life values are indicated.

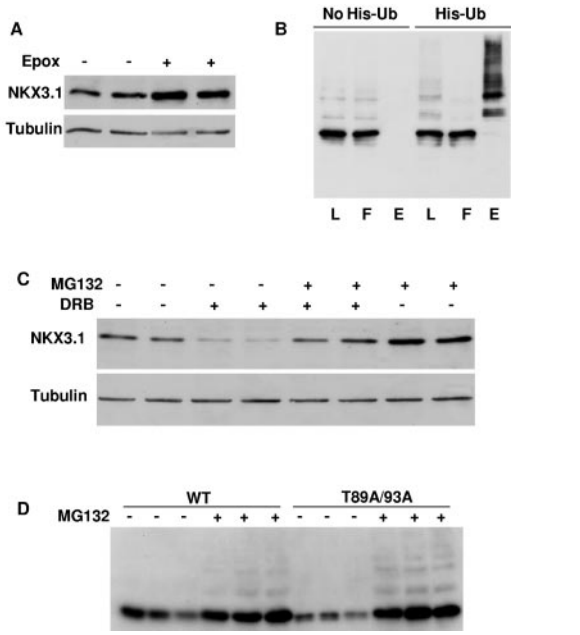

FIG. 5. NKX3.1 is degraded by the ubiquitin-proteasome system. (A) Western blot analysis to detect NKX3.1 in LNCaP cells treated with the proteasome inhibitor epoximicin (Epox). Two replicate wells were cultured in the presence or absence of epoximicin, and the whole-cell lysates were assayed with the antibodies indicated. (B) NKX3.1 is polyubiquitinated in vivo. NKX3.1 with empty vector and NKX3.1 with His-tagged ubiquitin (His-Ub) were cotransfected into LNCaP cells; 24 h after transfection, the cells were treated with MG132 for an additional 12 h and harvested in urea lysis buffer. The His-ubiquitin and ubiquitinated proteins were enriched by nickel resin chromatography and analyzed by Western blot using anti-NKX3.1 antibodies. L, lysate; F, flowthrough; E, eluate. Note that the discrete higher-molecular-weight forms of NKX3.1 are present in the lysate and eluate lanes. (C) Proteasome inhibition rescues the diminution of NKX3.1 induced by CK2 inhibition. Western blot analysis of NKX3.1 in LNCaP cells with or without DRB and MG132 treatment. DRB and/or MG132 was administered for 8 h and harvested for Western blot analysis using anti-NKX3.1 antibodies and antitubulin antibodies. (D) Proteasome inhibition increases the level of the T89A/T93A mutant. LNCaP cells in triplicate wells were transfected with the constructs indicated and exposed to MG132 for 12 h at 24 h posttransfection, followed by Western blot analysis using anti-NKX3.1 antibodies. The high-molecular-weight species that accumulate in the presence of MG132 represent polyubiquitinated isoforms of NKX3.1.

MG132 24 hours after transfection. Consistent with the interpretation that CK2 phosphorylation protects NKX3.1 from proteasomal degradation, the T89A/T93A mutant protein accumulated to a level similar to that of the wild-type protein in the presence of MG132 (Fig. 5D).

Phosphorylation of NKX3.1 by endogenous CK2α′ in LNCaP extracts. To determine whether CK2 and/or other kinase activities present in LNCaP cells were capable of phosphorylating NKX3.1, in-gel kinase assays were performed using a fractionated extract with recombinant human (NKX3.1) or mouse (Nkx3.1) protein as the substrate. Figure 6A shows the results of in-gel kinase assays of fractions 9 to 19 of a representative fractionation using either recombinant mouse or human protein as a substrate. Comparison of the signal obtained from gels containing NKX3.1 or Nkx-3.1 with a negative control gel (without substrate) revealed the presence of several kinase activities in fractions 9 to 19 with the ability to phosphorylate both forms. Prominent among these were several activities in the 40- to 45-kDa range that were readily apparent in the experimental gels but not in the negative control (Fig. 6A). One activity at approximately 44 kDa was observed only in the gel containing NKX3.1, and a second activity of approximately 42 kDa was present in gels containing either substrate.

To determine whether the kinase activity present in fractions 9 to 11 was coincident with the presence of either or both catalytic subunits of CK2, Western blot analyses were performed using subunit-specific antibodies. Western blot analysis of fractions 9 to 19 with anti-CK2α polyclonal antibodies demonstrated this subunit was not detectable in fractions 9 to 11, but was instead present in fractions 18 and 19 (Fig. 6B). In contrast, CK2α′ was present in fractions 9 to 11, in concordance with the kinase activity capable of phosphorylating NKX3.1. However, in fractions 18 and 19, where no 42-kDa kinase activity was observed by in-gel kinase assay, CK2α′ was also detected at a level similar to that observed in fractions 9 to 11 (Fig. 6B). To provide further evidence for the identity of the 42-kDa kinase, we attempted to immunodeplete CK2α′ from the early fractions. However, we were not able to identify an antibody that was capable of efficiently immunoprecipitating CK2α′ (data not shown). In lieu of immunodepletion studies, we opted to use pharmacologic inhibition of CK2 activity in in-gel kinase assays.

In the in-gel kinase assays performed in the presence of the kinase inhibitor DRB, the 42-kDa kinase activity present in fractions 9 to 11 was selectively inhibited in a dose-responsive

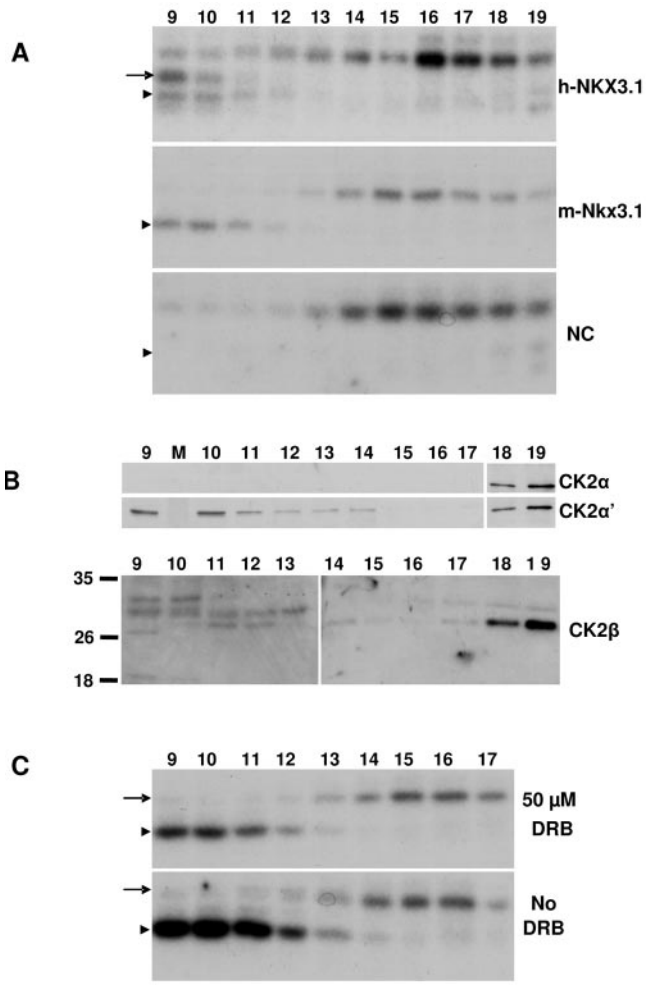

FIG. 6. NKX3.1 is phosphorylated by a 42-kDa kinase present in the LNCaP extract. (A) In-gel kinase assay of the Mono Q fractions of LNCaP lysate. Upper panel, human NKX3.1 as the substrate; middle panel, mouse Nkx3.1 as the substrate; lower panel, negative control with no protein cast in the gel. The arrowhead denotes the position of the 42-kDa kinase activity detected in gels with mouse and human NKX3.1 and absent in the control. The arrow denotes the position of the 44-kDa kinase activity detected only in the presence of human NKX3.1. Numbers indicate the fractions assayed. (B) Western blot analysis of fractions shown in panel A using the antibodies indicated. While using the anti-CK2β antibodies, cross-reacting species of other than 29 kDa were concentrated in fractions 9 to 13. M, molecular weight marker lane. (C) DRB selectively inhibits the 42-kDa kinase activity in an in-gel kinase assay. The arrowhead indicates the position of the 42-kDa kinase activity diminished in the presence of DRB. The arrow indicates the position of a 60-kDa kinase activity that is not inhibited by DRB and serves as a control for specificity.

manner (Fig. 6C and data not shown). Although DRB also inhibits CK1 and CK2α, neither of these has an apparent molecular mass of 42 kDa on SDS-PAGE gels. Taken together with the Western blot analyses, these data are consistent with the interpretation that the 42-kDa kinase capable of phosphorylating NKX3.1 is CKα′. These data also indicate that at least two forms of CK2α′ are present in the fractionated LNCaP extracts: one that elutes at a lower salt concentration that can phosphorylate NKX3.1 in an in-gel kinase assay, and a form that elutes at a higher NaCl concentration that cannot. The later-eluting form copurifies with CK2α and the regulatory subunit CK2β (Fig. 6B). These data suggest that so-called free CK2α′ that is not complexed in LNCaP cells with CK2α and CK2β can phosphorylate NKX3.1 in an in-gel kinase assay, whereas CK2α′ liberated from the holoenzyme cannot.

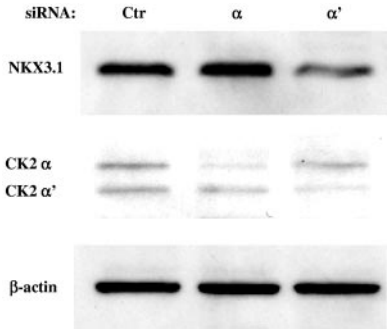

FIG. 7. CK2α′ knockdown by siRNA decreases the steady-state level of NKX3.1. LNCaP cells were transfected with siRNA oligonucleotides targeting CK2α or CK2α′ or a negative control (Ctr) siRNA as indicated. Levels of NKX3.1, CK2α, and CK2α′ were analyzed by Western blot using specific antibodies. β-Actin served as a control.

siRNA knockdown of CK2α′ diminishes NKX3.1 accumulation in LNCaP cells. Given that both apigenin and DRB inhibit other kinases in addition to CK2, an siRNA approach was used to decrease CK2α and CK2α′ activity in LNCaP cells. Transfection of LNCaP cells with a CK2α- or CK2α′-targeted siRNA led to a 70% decrease in CK2α or CK2α′ accumulation, respectively (Fig. 7). Interestingly, only cells in which CK2α′ was knocked down showed an ~50% decrease in the steady-state level of NKX3.1 (Fig. 7). These data suggest that in LNCaP cells, CK2α′ plays a central role in maintaining the level of NKX3.1.

DISCUSSION

Evidence from clinical samples and animal model systems points to NKX3.1 as a dose-dependent regulatory protein involved in prostate tumor initiation (48). Although considerable effort has been put forth to characterize the genetic mechanisms that underlie the decrease in NKX3.1 expression in human tumors, little is known about the biochemical mechanisms that operate to maintain expression of this prostate growth-regulatory protein. The data presented here identify protein kinase CK2 as a key regulator of NKX3.1 expression that operates at multiple levels in LNCaP cells. Our observations also show that functionally distinct isoforms of the CK2α′ subunit exist in these cells.

CK2 is a highly conserved dual-specificity kinase that does not appear to be directly coupled to any of the canonical signal transduction systems that tightly regulate the activity of most kinases (36). As such, it has often been referred to as a spontaneously active kinase (42). However, the growing number of key cellular processes that are affected by CK2 leave little doubt that its activity must be tightly regulated within the cell (4). First described over 50 years ago, CK2 (formerly casein kinase II) is among the best-characterized human kinases (12). CK2 exists as a heterotetrameric complex consisting of two catalytic subunits associated with two molecules of the regula-

tory subunit CK2β. Within the tetramer, the catalytic subunits can be homo- or heterodimers of CK2α and CK2α′. CK2β, which is not required for enzymatic activity, functions in the assembly of the tetrameric complex and may affect substrate specificity as well. CK2 tetramers are remarkably stable, and the assembly of the complex is a likely point of regulation (5, 39).

Increasing evidence suggests that CK2α and CK2α′, which resides outside of the tetrameric complex, not only have catalytic activity but may also have a different repertoire of phosphorylation substrates than the holoenzyme (39). The regulation of these two distinct pools of CK2 activity, the tetrameric forms and the so-called free forms, is currently not well understood. The data presented here provide new evidence that support the suggestion that these distinct pools may be functionally distinct. Fractionation by anion exchange chromatography readily separated free from complexed CK2α′ in LNCaP cell extracts, and the free form was able to phosphorylate NKX3.1 in in-gel kinase assays, while CK2α′ that cofractionated with CK2α and CK2β was not. These data indicate that free CK2α′ in LNCaP cells is functionally distinct from the complexed form by virtue of the fact that it displays a differential ability to phosphorylate NKX3.1.

Posttranslational modification of CK2α′ could play an important role in its inclusion in or exclusion from the tetrameric holoenzyme as well as the observed differential ability to phosphorylate NKX3.1. It is important to note that the studies reported here cannot exclude the possibility that CK2α′ in the context of an intact holoenzyme in vivo may be able to phosphorylate NKX3.1. In this scenario, substrate recognition by CK2α′ could be influenced by the β regulatory subunit or a CK2α heterotetrameric partner.

The only form of CK2α detected by Western analysis of LNCaP extracts cofractionated with CK2α′ and CK2β, and this form did not phosphorylate NKX3.1 in in-gel kinase assays. This form of CK2α remained catalytically active after liberation from the holoenzyme, since it was able to phosphorylate substrates other than NKX3.1 in in-gel kinase assays (data not shown). Since the Western blot analyses of fractionated extracts did not reveal the presence of free CK2α in LNCaP cells, we were not able to determine whether this isoform can phosphorylate NKX3.1. Addressing this question would require altering the level of CK2α by transfection or by expressing NKX3.1 in a heterotypic cell line that has free CK2α.

Blocking CK2 activity led to a sharp decrease in NKX3.1 expression in LNCaP cells by affecting both the level of mRNA and the half-life of the protein. At the RNA level it is not clear whether blocking CK2 results in a change in the rate of NKX3.1 transcription or in the stability of the NKX3.1 mRNA or both. Inhibition of the proteasome with either MG132 or epoximicin led to a significant increase in the level of NKX3.1 in LNCaP cells under standard culture conditions and reversed the decline in NKX3.1 accumulation observed when CK2 activity was blocked.

Taken together with the in-gel kinase and Western blot analyses, these data are consistent with a model in which phosphorylation by free CK2α′ protects NKX3.1 from degradation by the proteasome. This model is also consistent with the analyses of CK2 phosphorylation site mutants which demonstrated that replacement of Thr89 and Thr93 with alanines generated a less-stable isoform of NKX3.1. The fact that siRNA knockdown of CK2α′ but not CK2α led to a decrease in NKX3.1 accumulation further supports this model.

CK2 activity has been implicated in the control of cell differentiation, proliferation, and, more recently, cell death (4, 24, 36, 42). Hundreds of proteins have been suggested to be direct targets of CK2 phosphorylation, but only a few have been demonstrated to be phosphorylated on CK2 sites in vivo (36). A key function of CK2 appears to be the control of transcription factor activity, including homeodomain proteins. CK2 phosphorylation has been shown to regulate the DNA binding activity of Engrailed, Nkx2.5, SIX1, and CUT and to alter the ability of Antennapedia and Even-skipped to interact with binding partners but has not been implicated in homeodomain protein turnover (10, 17, 21, 27, 29, 35). However, CK2 phosphorylation of c-Myc and β-catenin results in stabilization by antagonizing proteasome-dependent degradation (13, 50). The data reported here strongly suggest that CK2 also regulates the level of NKX3.1 by preventing proteasome-mediated degradation.

NKX3.1 belongs to an expanding group of proteins that appear to function as haploinsufficient tumor suppressors (16, 20, 44, 45). *NKX3.1* maps to human chromosome 8p21, a region that undergoes frequent loss of heterozygosity early in the course of prostate cancer, and loss-of-function mutations in the mouse *nkx3-1* gene lead to prostate epithelial hyperplasia, even in heterozygous mice (2). Consistent with the concept of haploinsufficeincy, in human prostate cancer, the coding region of the remaining *NKX3.1* allele does not become mutated, and its transcript accumulates (41, 55). However, the abundance of the NKX3.1 protein has been reported to be diminished in prostate tumors in a manner that correlates with disease progression (11, 31). These observations underscore the need to elucidate both transcriptional and posttranscriptional modes of NKX3.1 regulation. *NKX3.1* is known to be androgen responsive, but other aspects of its regulation remain undefined. The experiments reported here identify CK2 as a critical NKX3.1 regulator in an androgen-responsive prostate tumor cell line.

Our observations raise the possibility that altered CK2 function may play a role in prostate cancer progression by affecting the expression of NKX3.1. In this scenario, CK2 activity would be predicted to be diminished in prostate tumor cells compared to normal prostate epithelial cells. In fact, using a peptide substrate to measure total CK2 activity in tumor samples, the level of CK2 has been reported to be significantly increased in prostate cancer cases (60). These observations would appear to be at odds with the predicted effect of CK2 on NKX3.1 expression. However, the data reported here demonstrate the need to distinguish between the activity of free versus complexed forms of CK2 in prostate tumors, since only the free form of CK2α′ can efficiently phosphorylate NKX3.1. To date, this question has not been addressed for prostate or any other tumors.

Interestingly, while total CK2 activity increases in prostate tumors, the level of nuclear CK2 decreases in prostate tumor cells, although the antibody used in this study did not distinguish between CK2α and CK2α′ (60). However, given that NKX3.1 is predominantly a nuclear protein, this observation is consistent with a model wherein diminished free CK2α′ expression could account, at least in part, for the epigenetic loss of NKX3.1 expression observed in many prostate tumors.

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